Dual Target of EGFR and mTOR Suppress Triple Negative Breast Cancer Cell Growth Via Regulation The Phosphorylation of mTOR Downstream Proteins

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Abstract

Background Triple-negative breast cancers (TNBC) are the most aggressive subtype of breast cancer, accounting for 15% - 20% of all cases, and have no response to available hormonal therapies and anti-HER2-targeted therapies due to the absence of corresponding targets. Over half of TNBC patients have overexpressed EGFR, but they are insensitive to EGFR inhibitors from monotherapy. Mammalian target of rapamycin (mTOR) connected with EGFR in the downstream signaling and involved in the progress of TNBC. The purpose of this study is to determine the combined effect of everolimus and gefitinib in a TNBC cell model and investigate the possible mechanism.

Results: This work showed the expression EGFR and p-mTOR protein in TNBC tissues were significantly higher than that in non-TNBC ($p < 0.05$), while the expression of mTOR, S6K1, pEGFR and p-S6K1 were significantly higher in the EGF stimulation. EGFR and p-mTOR protein are related to poor prognosis. EGFR inhibitor gefitinib and mTOR inhibitor everolimus significantly inhibited the proliferation of human triple-negative breast cancer MDA-MB-468 cells and arrested cells in G0/G1 phase when applied separately and in combination in a dose-dependent manner ($P < 0.05$). Meanwhile, the rate of apoptosis of MDA-MB-468 cells was significantly increased separately by two drugs ($P < 0.01$). Furthermore, the combination of everolimus and gefitinib reduced the phosphorylation of mTOR downstream proteins. Instead, the phosphorylation of 4E-BP1 was enhanced after the everolimus and gefitinib treatment, indicated an alternative activation pattern.

Conclusions: These results suggested that dual inhibition of mTOR and EGFR could be a promising approach to treat TNBC.

Introduction

Breast cancer is one of the most common malignant tumors in women. The incidence rate of breast cancer ranks first in Europe, America and the eastern areas in China (1). Although the total mortality rate has been declining due to improvements in diagnostic techniques and refinements in treatment modalities in many developed nations in the past decades, the mortality caused by advanced types of breast cancers remained at a high level. Most patients are presented with the unresectable disease and current treatment options of chemotherapy and radiotherapy are unsatisfactory. New strategies are needed in the treatment of this disease in order to improve patients' survival rate.

Breast cancer is known as highly heterogeneous, different types of breast cancers show considerable heterogeneity despite a common tissue of origin. In clinical, breast cancer were usually divided into several subtypes according to the status of several molecular biomarkers (2): estrogen receptor (ER) or progesterone receptor (PR) positive (also known as Luminal type), human epidermal growth factor receptor 2 positive with or without ER and PR positivity (also known as HER2 overexpressed type) and triple-negative breast cancer (TNBC) defined by the absence of ER/PR expression and HER2 amplification. Among them, TNBC is the most aggressive subtype accounting for 15% - 20% of breast

cancer and encompasses more than one molecular subtype. In contrast to ER/PR positive and (or) HER-2 negative molecular subtypes, TNBC has the clinical characteristics of being rapidly progressive, earlier onset of metastatic and no response to available hormonal therapies and anti-HER2-targeted therapies (3). Currently, chemotherapy and radiation therapy are the only ways to treat TNBC patients (4, 5), but the prognosis is poor due to drug resistance and recurrence in the first five years (6). Thus, it is urgent to develop new therapeutic targets for TNBC patients.

Epidermal growth factor receptor (EGFR) belongs to a large family of cell surface receptors. In normal cells, the expression of EGFR is estimated to be 40000-100000 receptors per cell (7). In cancer cells, over expression of more than 106 receptors per cell was observed (8). The activation of EGFR requires ligand binding. The binding of the EGF ligand to the EGFR extracellular domain (EGFR-ECD) promotes its inactive-to-active conformational transition (activation), the functional conformational transition of EGFR-ECD is a consequence of the cooperative motion of protein domains driven by the EGF ligand binding(9). When the ligand binds to the extracellular region of EGFR, the receptor forms a dimer to activate its kinase activity, and then autophosphorylates at multiple tyrosine residues in the intracellular region to recruit various substrates. As an important ligand of EGFR, EGF can stimulate cell proliferation, differentiation, growth, migration and inhibit apoptosis. Previous studies have shown that the addition of EGF to HeLa cells can activate EGFR, resulting in the phosphorylation of all 2244 proteins at 6600 sites (10). In addition, EGF stimulation caused significant differences in the expression of 3172 genes and 596 proteins in human breast epithelial cells (HMEC) (11). Receptor activation promotes cell proliferation, motility and survival by activating various downstream signaling pathways, such as Ras-Raf-MAPK and mTOR-PI3K-AKT pathways(12). EGFR plays an important role in the occurrence and development of a variety of malignant tumors. It can promote the growth, proliferation, angiogenesis, invasion, metastasis and apoptosis of tumor cells.

Clinical data have been shown that more than half of TNBC have EGFR overexpression, and EGFR overexpression often associates with poor prognosis (13, 14). This suggests that TNBC patients are likely to benefit from EGFR targeted therapies, and several EGFR inhibitors are under evaluation in clinical. Unfortunately, the commonly used inhibitors cetuximab and gefitinib which are effective in NSCLC failed to demonstrate significant efficacy in TNBC when used alone (15, 16). The possible reason for its inefficiency might be the activation of EGFR-dependent signalling pathways, especially the PI3K/AKT/mTOR pathway in EGFR overexpressed TNBC. The mammalian target of rapamycin (mTOR) is a serine/threonine kinase that is directly phosphorylated and activated by AKT. Activation of mTOR phosphorylates of eukaryotic translational initiation factor 4E-binding protein (4E-BP1) and ribosomal protein S6 kinase (P70S6K), which leads to protein translation and tumor growth (17, 18). Preclinical studies have suggested that targeting mTOR could improve the efficacy of EGFR inhibitors in various human cancers, including TNBC, mTOR inhibitor rapamycin combined with paclitaxel can affect the PI3K / Akt / mTOR signaling pathway and improve the therapeutic effect of triple negative breast cancer (19).

Given the connection between EGFR and mTOR pathway in TNBC, we hypothesised dual-targeting of mTOR and EGFR could be a potential therapeutic strategy to treat TNBC. In the current study, we

examined the expression of EGFR and mTOR downstream proteins in TNBC tissue samples and investigated the combinational effect of everolimus and gefitinib in TNBC cell line.

Material And Method

Patients and tissue samples. The 111 female patients in Affiliated Tumor Hospital of Xinjiang Medical University of China were recruited to the study during January to June in 2013, and meanwhile, the clinical and pathological data of these patients were collected as well. Authors had been permitted to access to information that could identify individual participants during or after data collection. The median age of patients was 47.5 years (range 29-72 years), in which 81 patients have 0-3 lymph nodes metastasis and 30 patients have ≥ 4 lymph nodes metastasis. All patients underwent surgery for breast cancer at the department of mammary gland head and neck surgery. After surgery, tissue samples were fixed in 7.5% buffered formalin and embedded in paraffin for routine diagnostics. According to the International Union Against Cancer (UICC) 2004 issue of the TNM system, there were 43 patients with clinical stage I, 46 patients with clinical stage II and 22 patients with clinical stage III. In terms of expression of ER, PR, HER-2 and Ki-67, all patients were divided into 42 luminal, 48 TNBC and 21 HER-2 overexpression subtypes. Among them, 48 TNBC patients were followed up to 60 months for 5-year survival records. The detail of patients information was presented in Table 1. All protocols were approved by Xinjiang Medical University and informed consent was obtained from these patients.

Immunohistochemistry (IHC). Tumor tissue derived from surgical specimens were fixed in 7.5% formalin and performed IHC on 4 mm paraffin sections from one representative tissue block per patient following the manufacturer's instructions. The sections were stained mAb against EGFR, phospho-mTOR (p-mTOR), phospho-4eEBP1 (p-4eEBP1) and phospho-S6K1 (p-S6K1) (1:100 dilution, Cell Signaling), then counterstained with hematoxylin. The protein expression was detected using a Horseradish peroxidase (HRP) conjugated rabbit anti-mice IgG secondary antibody, followed by colorimetric detection using DAB. The positive expression was determined under microscope ($\times 400$) by the clear brown granules in the membranous or cytoplasm and/or nucleus. Five fields were randomly selected and a total of 200 cells were counted. The intensity of staining was scored - for colorless, + for light yellow, ++ for yellow and brown yellow. The positive index scored negative (-) when the number of positive cells less than 10%, weak positive (+) when the number of positive cells were 10% - 50%, and strong positive (++) when the number of positive cells were more than 50%.

Cell Lines and Reagents. MDA-MB-468 cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in DMEM medium supplemented with 10% heat-inactivated foetal bovine serum (FBS) and 1% PS at 37°C in a humidified atmosphere of 5% CO₂. The EGFR tyrosine kinase inhibitor Iressa (gefitinib) and the mTOR inhibitor everolimus were purchased from APExBIO. EGF was purchased from GIBCO. Drugs were dissolved in DMSO and stored at -20°C. Dilutions were made immediately before use in growth medium. Cell Counting Kit-8 was purchased from TransGen, and FITC Annexin V Apoptosis Detection Kit from BD(USA). The primary rabbit mAb to mTOR(2972), Phospho-mTOR(Ser2448)(2971), 4E-BP1(9452), Phospho-4E-BP1 (Thr37/46) (2855), p70 S6

Kinase(9202),Phospho-p70 S6 Kinase (9205), EGFR(2232),Phospho-EGFR(D7A5) XP® (3777) were purchased from Cell Signaling (USA). Anti-ACTB rabbit polyclonal antibody(D110001) from Shanghai Sangon Biotech Inco. Goat anti-rabbit IgG H&L (HRP)(ab205718) from Abcam. All other reagents were purchased from Sigma (St. Louis, MO, USA) unless indicated.

Western blot. Immunoblotting was performed as described previously. Cells were cultured in 100 mm dishes at a density of 5×10^5 cells per dish briefly and treated with indicated drugs for 1 h or 2 h. Twenty micrograms of each protein samples from cell lysates were separated via 7-12% SDS-PAGE and transferred to PVDF membranes. Blots were blocked and incubated overnight at 4°C with the indicated primary antibodies. All primary antibodies were diluted at 1:500 except antibodies against mTOR,S6K1 and p-S6K1 at 1:1000. The immunoreactive proteins were detected using the enhanced chemiluminescence method and quantified using Image J software. The intensity of individual bands was expressed relative to the control signal.

CCK-8 assay. MDA-MB-468 Cells (5×10^4 cells per well) in the logarithmic phase were plated in 0.5 ml complete medium in 96-well plates and allowed to attach overnight. The next day, Iressa and everolimus or their combination were added at concentration as indicated. Cells were incubated for 48 h at 37 °C in a humidified atmosphere containing 5% CO₂. Afterwards, the cells were added 10% of CCK-8 solution and allowed to incubate another 1 h. Absorbance was recorded by a Bio-Rad xMark™ plate reader at 4500 nm. All experiments were run in triplicates.

Cell cycle analysis, cell proliferation and apoptosis. Cells were cultured and treated with drugs as described above. After 48 h of incubated with three treatment, cells were harvested and fixed with 80% cold ethanol overnight. The next day, after centrifugation with 2500 rpm for 5 min, the cell pellets were resuspended with PI/RNase staining buffer and filtered with 200 mesh nylon sieve to form single cell suspension. Cell cycle analysis was performed using a BD FACS Aria™ flow cytometer and cell cycle distribution was calculated using software. To further analyse apoptosis, cells treated with indicated drugs were harvested and performed AnnexinV-FITC and PI staining for 15 min at room temperature in the dark. Cells were analysed by flow cytometry, and the results were expressed as the percentage of apoptotic cells, including both early (annexin V-positive, PI negative) and late (annexin V-positive, PI positive) apoptotic cells, relative to the total number of cells.

Statistical analysis. All experiments were performed at least in triplicate and repeated three times. Unless otherwise indicated, the results are expressed as the mean $\pm$ standard deviation (SD) format. Statistical analysis was performed by SPSS 19.0. The results from the different groups were compared by one-way analysis of variance (ANOVA). Kaplan Meier method was used for survival analysis. For all statistical analyses, differences were considered to be statistically significant at $P < 0.05$.

Results

Expression of EGFR, p-mTOR, p-S6K1 and p-4EBP1 in TNBC. The expression of EGFR, p-mTOR, p-S6K1 and p-4EBP1 in BC patients were analysed by immunohistochemistry on 48 patient tissue samples. Overall, the expression of four proteins were positively correlated with lymph node metastasis and clinical stage ($P < 0.05$). The positive rate of EGFR, p-mTOR, p-S6K1 and p-4EBP1 expression in patients with lymph node metastasis ≥ 4 were 66.7%, 63.3%, 60.0% and 70% respectively, which were significantly higher than those in patients with lymph node metastasis 0-3 ($P < 0.05$). (Supplement table S1) Moreover, the expression rate of EGFR, p-mTOR, p-S6K1 and p-4EBP1 at stage I to stage IV were remarkably increased from 0.04%, 20.9%, 14.0% and 25.5%, to 61.5%, 63.6%, 63.6% and 68.2% respectively. (Supplement table S2) According to the expression of ER, PR, HER-2 and Ki-67, the included 111 breast cancer patients were divided into three types: Luminal (42), TNBC (48) and HER-2+ types (21). The expression rate of EGFR, p-mTOR, p-S6K1 and p-4EBP1 in Luminal, TNBC and HER-2+ types were 19.0%, 23.8%, 26.2%, 28.6%; 43.8%, 52.1%, 39.6%, 41.7% and 23.8%, 19.0%, 28.6%, 26.2% respectively. The expression rate of EGFR and p-mTOR in TNBC were significantly higher than that in luminal type and HER-2+ type ($P < 0.05$) (Supplement table S3). The representative images for immunostaining and immunohistochemistry of TNBC tissues and their adjacent tissues are shown in Figure 1. EGFR showed cytoplasmatic and membranous staining pattern, p-mTOR was expressed cytoplasmatic, p-S6K1 and p-4EBP1 were expressed in cytoplasm and/or nucleus, whereas four proteins were negative or weak expressed on adjacent tissues.

Correlation of EGFR, p-mTOR, p-S6K1 and p-4EBP1 expression with TNBC patients Survival. The 48 TNBC patients were followed up to 60 months for analysis the correlation between survival and the expression of EGFR, p-mTOR, p-S6K1 and p-4EBP1. The Kaplan Meier survival analysis of TNBC patients showed the average 5-year DFS in EGFR positive group (43.15 months) and p-4EBP1 positive group (47.16 months) were significantly shorter than those in negative groups (54.66 and 51.78 months respectively), otherwise, there was no significant difference between p-mTOR positive group (49.4 months) and p-S6K1 (48.47 months) compared with their negative groups (50.42 and 50.79 months respectively) (Fig. 2). The results indicated that overexpression of EGFR and p-4EBP1 in TNBC patients might predict a poor prognosis.

Expression EGFR, p-mTOR, p-S6K1 and p-4EBP1 in EGFR-mTOR signaling by EGF induction. The MDA-MB-468 is a TNBC cell line. For evaluating the downstream signaling of EGFR-mTOR, MDA-MB-468 cells were treated with two concentrations of EGF in two separate runs. Western blot results showed the expression of mTOR, p-mTOR, p-S6K1 and p-EGFR in 10 ng/ml as well as 100 ng/ml of EGF treatment were significantly increased than those without EGF treatment ($p < 0.05$). On the contrary, the expression of p-4EBP1 in EGF treatment was significantly decreased than those without EGF treatment ($p < 0.05$) (Fig. 3). There was no significant difference between two concentrations and two different tests ($p > 0.05$). This result showed the activation of EGFR-mTOR signaling in MDA-MB-468 cells.

The EGFR and mTOR inhibitors are synergistic in TNBC cell. For evaluating the effects of EGFR and mTOR inhibitor on the growth of TNBC cell line MDA-MB-468, Iressa and Everolimus were used to test cell proliferation individually and collaboratively in CCK-8 assay. The results showed EGFR inhibitor Iressa and mTOR inhibitor Everolimus significantly inhibited the proliferation of MDA-MB-468 cells in a dose-

dependent manner used separately or in combination ($P < 0.05$). The growth inhibitory value at 50% (IC_{50}) for Iressa and Everolimus were 20.470 ± 5.23 and $20.192 \pm 6.10 \mu M$ respectively. For evaluating synergy of the two-drug combination, different concentration of combinations were used to test the inhibitory effects on MDA-MB-468 cells. The result showed each combination significantly inhibited cells compared with untreated control ($P < 0.01$) (Fig. 4). According to the formulation: combined effect $Q = \text{inhibitory rate of combination} / [\text{inhibitory rate of drug A} + (1 - \text{inhibitory rate of drug A}) \times \text{inhibitory rate of drug B}]$, the Q value of $10 \mu M$ Iressa combined with $10 \mu M$ was 1.097, indicating the synergistic effects.

Effects of EGFR and mTOR inhibitor on the cell cycle and apoptosis. The inhibition of cancer cell generally resulted in cell growth arrest. Subsequently, we examined the cell cycle and apoptosis of MDA-MB-468 cells treated with Iressa and Everolimus by flow cytometry. The results showed that $71.19 \pm 1.476\%$ of Iressa treated cells, $73.337 \pm 0.55\%$ of Everolimus treated cells and $78.273 \pm 0.34\%$ of cells treated in combination are in G0/G1 phase, which is significantly higher than that in untreated cells ($52.487 \pm 0.416\%$) ($P < 0.01$), and the proportion of cells in G0/G1 phase treated in combination is significantly higher than that in cells treated individually ($P < 0.05$). The results indicated that both Iressa and Everolimus and its combination arrested MDA-MB-468 cells at G0/G1-phase (Fig. 5).

For evaluating the effects of EGFR and mTOR inhibitors on the cell death, we analyzed apoptosis of MDA-MB-468 cells with Annexin V/PI assay after Iressa and Everolimus were treated individually and in combination. The results showed both Iressa or Everolimus alone and their combination significantly induced apoptosis of MDA-MB-468 cells ($P < 0.01$), the apoptosis rate induced by Iressa or Everolimus and their combination were 12.2%, 11.3% and 10.6% respectively (Fig. 6). However, there was no significant difference between the treatment in combination and the treatment applied individually ($p > 0.05$). This indicates there is no synergistic effect between the two drugs in apoptosis induction.

Effects on EGFR and mTOR pathways after treatment of MDA-MB-468 cells with EGFR and mTOR inhibitors. To assess the effects of iressa and everolimus on the EGFR/mTOR signalling pathways, we analysed the protein expression of total and phosphorylated forms of EGFR, mTOR, S6K1 and 4EBP1 by Western blotting. MDA-MB-468 cell lines were treated with iressa and everolimus as single agents and in combination. There were no significant difference of EGFR expression in each treatment. Compared with untreated, both iressa alone and in combined with everolimus significantly decreased protein expression of mTOR, p-mTOR and p-SK61 ($P < 0.05$). However, everolimus has no effects on mTOR and p-mTOR, but markedly decreased the expression of p-SK61. Compared with single agents, the combination only significantly decreased the expression of p-mTOR. In contrast to predict, the expression of p-4EBP1 significantly increased in single agents and in combination ($P < 0.05$) (Fig. 7, Supplement table S4).

Discussion

One of the remarkable characteristics of triple negative breast cancer is EGFR overexpression and dysregulation of the PI3K/AKT/mTOR signalling pathway. Researchers are now seeing encouraging clinical activity from molecularly targeted approaches to TNBC. As the failure of single agents for

targeted therapy, combined therapeutic strategy such as the combination of EGFR and mTOR inhibitors is a reasonable choice for the treatment of TNBC.

In the present study, we firstly confirmed the expression of EGFR, p-mTOR, p-S6K1 and p-4EBP1 in the clinical samples from TNBC patients and the activation of EGFR-mTOR signaling in MDA-MB-468 cell line. The EGFR was overexpressed in 43.8% TNBC patients which is significantly higher than that in Luminal and HER-2+ types and consistent with what was reported previously (20, 21). From the survival analysis, we deduced that overexpression of EGFR and p-mTOR is related to the poor prognosis of TNBC. It indicated that EGFR and mTOR play important role in development of TNBC and have potential to be exploited as targets. Similar to other studies (22–24), our work found that combined treatment with iressa and everolimus had a synergistic effect on inhibiting TNBC cell growth and induced MDA-MB-468 cell apoptosis as well as cell cycle arrest at G0/G1-phase. In the combined drug experiment, it can not be ignored that after exploring the intervention concentration of single drug group and combined drug group, the cell experiment concentration of EGFR inhibitor gefitinib and mTOR inhibitor everolimus in our single drug group reached 10 μ M. However, the drug concentration in the combination group was Iressa 0.5 μ M + Everolimus 0.5 μ M. The drug concentration difference of 20 times between the single drug group and the combination group showed similar inhibitory effect on triple negative breast cancer cells, which indicated that the combination of the two drugs could achieve similar therapeutic effect with the high-dose single drug in the case of very small dose combination, but the side effects caused by the high and low dose of drugs and the tolerance of patients to drugs were very different, Therefore, if it can be confirmed in clinical trials, patients receiving treatment can achieve the same therapeutic effect as high-dose monotherapy in the case of low-dose combination, but can greatly reduce the side effects of drugs. The combination markedly decreased the expression of mTOR and p-mTOR, suggested a negative feedback inhibiting effect in mTOR signaling and decreased phosphorylation of mTOR downstream proteins.

Although overexpression of EGFR is frequent in TNBC, monotherapy with EGFR inhibitor hardly give positive response. As proposed by others that anti-EGFR agents alone mostly inhibited the RAS/MAPK signalling pathway more effectively than the PI3K/AKT/mTOR pathway in TNBC (22–24). At present, there are few cases of targeted drugs used in clinic alone, except gefitinib for non-small cell lung cancer, Gleevec for stromal tumor, sorafenib for renal cancer. Based on the current domestic and foreign studies on the clinical application of targeted drugs, the effective rate of a single targeted drug on tumor is very limited, and its effective rate is about 10% (25). Targeted therapy has its advantages, such as high selectivity and specificity, but it also has the limitation that its anti-tumor effect is generally mild. It is difficult to achieve satisfactory therapeutic effect with single drug in clinic. Therefore, the combination of various targeted cell pathway inhibitors may open up new therapeutic means for the treatment of refractory tumors (26, 27). EGFR is overexpressed in a variety of malignant tumors, and mTOR signaling pathway is in a central link of multiple signaling pathways. As its upstream protein, EGFR plays a regulatory role in mTOR signaling pathway. A number of preclinical studies have been carried out abroad on the combined use of EGFR and mTOR pathway inhibitors, such as in non-small cell lung cancer, brain glioma, pancreatic cancer, biliary tract cancer and breast cancer. However, up to now, the combined

application of EGFR and mTOR inhibitors remains in the preclinical study, and there is no clinical combination. Therefore, it is the direction of future researchers to find the mechanism and evidence of improving the efficacy of the combination of EGFR and mTOR inhibitors. our study provided evidence that mTOR downstream signaling proteins were highly activated in TNBC. This suggested combined mTOR inhibitors can sensitize TNBC to EGFR inhibitors.

mTOR comprised two structurally and functionally distinct multiprotein complexes, TORC1 and TORC2. Its phosphorylation activated downstream proteins and involved in cancer development. In our study, the mTOR signalling proteins were phosphorylated by the EGF stimulation, and nearly all signalling proteins were de-phosphorylated after EGFR and mTOR inhibitors treatment. It might be one of the mechanisms of inhibiting TNBC cell growth by two inhibitors.

Surprisingly, in contrast to down-regulation of the p-4EBP1 expression which reported in previous studies (22), the expression of p-4EBP1 was significantly increased after iressa and everolimus treatment done individually and in combination. This raise the question of what is the role of 4EBP1 in mTOR signalling. 4EBP1 is a tumor suppressor, its inactivation (phosphorylation) may involve in the development of some tumors and induce epithelial mesenchymal transition, migration and invasion of cancer cells (23, 24, 28). Therefore, the expression of p-4EBP1 in tumor cells may indicate its carcinogenic potential. Phosphorylated 4EBP1 is generally considered as a marker of activated mTOR signaling. Recently, several kinases were found to phosphorylate 4EBP1 in mTOR dependent or independent way, suggesting that mTOR may not be the only kinase that phosphorylates 4EBP1 (29). The resistance of mTOR inhibitors is related to their inability to block 4EBP1 phosphorylation in some cancers (30–35). Together with the above mentioned, we deduced the 4EBP1 might be phosphorylated by alternative kinase, and not influenced by the p-mTOR. Whether there exists novel activation mechanism on 4EBP1 deserve further study.

Conclusions

In conclusion, we found that the EGFR and mTOR pathways are active and coexpressed in a significant proportion of TNBC patients. A combination of EGFR and mTOR inhibitors showed a synergistic tumor inhibitory effect, which might be mediated via regulation the phosphorylation of mTOR downstream proteins. Our study suggested that dual inhibition of mTOR and EGFR could be a promising approach to treat TNBC.

Abbreviations

EGF: Epidermal growth factor

EGFR: epidermal growth factor receptor

TNBC: triple negative breast cancer

mTOR: mammalian target of rapamycin

RPS6K: Ribosomal protein S6 kinase

4EBP1: 4E binding protein 1

HER2: human epidermal growth factor receptor 2

ER:estrogen receptor

PR: progesterone receptor

NSCLC: non-small cell lung cancer

IHC: Immunohistochemistry

DAB: 3,3'-diaminobenzidine

HRP:Horseradish peroxidase

DMEM:dulbecco's modified eagle medium

MAPK:Mitogen-activated protein kinase

PI3K: Phosphoinositide-3 kinase

DMSO: Dimethylsulfoxide

FITC: Fluorescein isothiocyanate

CCK-8: Cell Counting Kit-8

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophores

PVDF:Polyvinylidene fluoride

Declarations

- Ethical Approval and Consent to participate

All protocols were approved by Xinjiang Medical University and informed consent was obtained from these patients.

- Consent for publication

Not applicable

- Availability of supporting data

Not applicable

- Competing interests

The authors declare that they have no competing interests.

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- Authors' contributions

Jing Ma and Binlin Ma conceived and designed this study. Hongtao Li , Yanzhen Cao and Jingjing Fan participated in the coordination and execution of the study. Jing Ma and Binlin Ma analyzed the data and interpreted of results. Jing Ma drafted the manuscript. Binlin Ma provided critical comments, suggestions and revised the manuscript. All authors read and approved the final version of the manuscript.

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Tables

Table 1. Clinical and pathological features of breast cancer patients

Baseline characteristics	
Total(n)	111
Histological classification	
Luminal	42 (37.8%)
TNBC	48 (43.2%)
HER-2+	21 (18.9%)
Age	
< 45	54 (48.6%)
≥45	57 (51.4%)
AJCC/UICC TNM classification	
Stage I	43 (38.7%)
Stage II	46 (41.4%)
Stage III	22 (19.8%)
Menarche	
< 14	45 (40.5%)
≥14	66 (59.5%)
Pregnancy	
< 3 times	53 (47.7%)
≥3 times	58 (52.3%)
Menopause	
Yes	50 (45.0%)
No	61 (55.0%)
lymph node metastasis	
0-3	81 (73.0%)
≥4	30 (27.0%)

Figures

Figure 1

Immunohistochemical staining of TNBC and their adjacents tissues for EGFR(A,B), p-mTOR(C,D), p-S6K1(E,F) and p-4EBP1(G,H) expression (all magnification $\times 400$). This figure is the representative of IHC of 48 TNBC patients tumor tissues and their adjacents tissues. The positive expression was determined under microscope ($\times 400$) by the clear brown granules in the membranous or cytoplasm and/or nucleus.

Figure 2

Disease-free survival curve of patients with triple negative breast cancer under different expression of EGFR(A) p-mTOR(B) p-S6K1(C) and p-4EBP1(D). The difference of 5-year DFS between positive and negative groups were tested by Log Rank and Breslow statistics, $p < 0.05$ was considered a significance difference.

Figure 3

Effects of EGFR and mTOR pathways after EGF treatment on MDA-MB-468 cells. (A) The expression level of mTOR, p-mTOR, p-S6K1 and p-EGFR in 10 ng/ml as well as 100 ng/ml of EGF treatment were significantly changed than those without EGF treatment ($p < 0.05$). (B) Western blotting of the expression patterns of EGFR and mTOR pathways protein.

Figure 4

MTT assay the effect of Iressa and Everolimus on MDA-MB-468 cell growth individually and collaboratively. The statistical analysis was presented on the inset table. ^Δ compared with blank group, $P < 0.01$; [▼] compared with Iressa 0.1 μM + Everolimus 0.01 μM, $P < 0.01$; [▽] compared with Iressa 0.01 μM + Everolimus 0.5 μM, $P < 0.01$; [▲] compared with Iressa 0.5 μM + Everolimus 5 μM, $P < 0.01$; [☆] compared with Iressa 10 μM + Everolimus 10 μM, $P < 0.01$.

Figure 5

MDA-MB-468 cell cycle arrested induced by Iressa and Everolimus. MDA-MB-468 cells were treated with PBS(blank), Iressa, Everolimus and their combination for 48 h and detected by flow cytometry.

Figure 6

FACS analysis of annexin V-FITC labeling of apoptosis in MDA-MB-468 cells after treatment with iressa and everolimus individually and in combination. (A) FACS spectra for apoptosis after the treatment of indicated drugs. (B) Quantitative data for apoptosis analysis. Mean $\pm$ SEM, n=3. Asterisks (**) designate significant differences from the control (0 μ M) at 0.01.

Figure 7

Western blot analysis of EGFR and mTOR pathways in MDA-MB-468 cell line treated with iressa and everolimus(A) and the relative expression according to grayscale scanning(B). * compared with control group, $P < 0.05$

Supplementary Files

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